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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this study is to evaluate the role of the growth factor Cripto-1 (CR-1) in mammary development and carcinogenesis. During the final year of this grant we developed adenoviral vectors for the expression of CR-1 and cloned CR-3 from MCF-7 cells. We overexpressed CR-1 and CR-3 in MCF-7, Vero, and HC-11 cells, and found a dramatic difference in phenotype of the transduced cells. This may indicate that the few differences present between CR-1 and CR-3 are in functional regions of the protein. We are currently developing GFP-free adenoviral vectors and viruses that only contain one of the differences between CR-1 and CR-3 to define which of the amino acid differences are responsible for the difference in phenotype of transduced cells.

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Randall Kinter 12/11/00

PI - Signature Date

Table of Contents

Introduction

The format for this report is as follows: selected sections of the introduction have been included for reference. Progress to date has been reported in indented paragraphs under the appropriate section of the proposal. Where appropriate, achievement of specific tasks in the statement of work has been addressed.

The incidence rate for breast cancer generally increases with advancing age [1]. It remains unclear which variables may be involved in this relationship, though one candidate is a newly discovered member of the epidermal growth factor family, Cripto-1 (CR-1) [2-4]. Previous results from our laboratory indicate that CR-1 expression is increased in old mammary tissue [5], and that treatment of old mammary cells with tamoxifen *in vitro* reduces CR-1 mRNA levels (our unpublished results). **These results suggest that CR-1 may be involved in carcinogenesis in older mammary tissue and also in the hormonal regulation of breast cancer.**

CR-1 is expressed at low levels in adult murine, spleen, heart, lung, brain, and breast [6, 7], and at elevated levels during fetal development [6] and during the growth of mammary epithelium during adolescence and pregnancy [7]. CR-1 is expressed in human breast cancer tissues [8, 9] and cell lines (both estrogen receptor positive and negative, [8]), as well as in colorectal tumors [10] and pancreatic cancer cells [11]. In two separate studies, 75-82% of breast cancer tissue samples were positive for CR-1, whereas 0-13% of adjacent, non-involved tissue samples were positive [8, 9], implying that CR-1 may be involved in growth regulation of breast cancer cells. In addition, over-expression of CR-1 in some immortalized murine mammary epithelial cells is sufficient to cause an increase in anchorage-independent growth, but not sufficient for tumor formation in nude mice [12]. Finally, treatment of CR-1-expressing teratoma cells with retinoic acid results in differentiation of the cells and shutoff of CR-1 transcription [2]. **All of the currently published data are correlative and provide only circumstantial evidence for a functional role of CR-1 in breast cancer.**

CR-1 plays a significant role in the neoplastic phenotype of some human colon cancer cell lines [13]. Treatment of CR-1-positive colon cancer cells with antisense RNA (either directly *in vitro* or by infection with a retroviral antisense expression vector) resulted in decreased CR-1 protein levels, reduction in both anchorage-dependent and -independent growth, and reduced ability to form tumors in nude mice [13]. **These results suggest that reduction, or better yet, elimination of CR-1 expression in CR-1-positive tumor cells may have a significant therapeutic effect.**

Hypotheses

1. Proper expression of Cripto-1 (CR-1) is required for the normal development of the mammary gland epithelial structure. (T.O. 1)
2. Overexpression of CR-1 will increase tumorigenicity of non-tumorigenic or moderately tumorigenic mammary epithelial cell lines. (T.O. 2)
3. Underexpression of CR-1 will decrease tumorigenicity of highly or moderately tumorigenic cell lines. (T.O. 2)
4. Efficient delivery of a ribozyme or other therapeutic gene to a developing tumor may be accomplished by injecting a retroviral vector at the tumor location. (T.O. 3)

5. Delivery of a retroviral vector containing a CR-1-specific ribozyme to an established CR-1-expressing tumor *in vivo* will result in the regression of that tumor. (T.O. 4)

Technical Objectives

1. Determine the role, if any, for CR-1 expression in the developing mouse mammary gland.
   - **a.** Infect progenitor cells of the mammary epithelial tree with CR-1 over- or under-expressing retroviral vectors; examine mammary epithelial morphology.
   - **b.** Quantify changes in CR-1 protein expression in the mammary gland.
2. Analyze the role of CR-1 in the neoplastic growth of murine mammary epithelial cell lines (CL-S1, -SA, and +SA) *in vitro* and *in vivo*.
   - **a.** Look for changes in *in vitro* growth rate and anchorage-independent growth in cells over- and underexpressing CR-1.
   - **b.** Examine tumor and metastasis production in syngeneic hosts *in vivo* in cells over- and underexpressing CR-1.
3. Establish a model system for the delivery of a gene to a mammary tumor using a retroviral vector.
   - **a.** Use the defective (non-replicative) retroviral vector, CA1, which expresses β-galactosidase (β-gal), to infect tumor cells transplanted into epithelium-free mammary fat pads. Determine both the optimum conditions for infection of tumor cells and the largest tumor size which can be effectively infected.
4. Use a retroviral vector for treatment of mammary tumors *in vivo*.
   - **a.** Use the retroviral ribozyme construct from T.O. 1 to treat mammary epithelial cell tumors. Generate mammary tumors and then use the optimum conditions determined in T.O. 3 for delivery of the ribozyme. Score alterations in tumor growth and the percentage of tumor and non-tumor cells infected.

Body

Results to date are indented from the previously submitted procedures and methods.

T.O. 1a. The CA1 retroviral vector (gift from P.A.W. Edwards, University of Cambridge) will be used for these experiments. The packaged vector will be produced in GP+E-86 cells, a mouse-specific retroviral packaging cell line [14]. Viral supernatants will be harvested, then filtered through 0.45 μm membranes to remove cells and cellular debris. If necessary, the virus may be concentrated by centrifugation through a 20% sucrose cushion at 34,000 x *g* for 6 hr, followed by resuspension in 1/100 of the original volume [15]. Viral titers will be determined by infection of +SA cells with serially diluted virus stocks, followed by staining with x-gal (or selection with G418). The number of blue cells (or $G418^R$ colonies) will indicate the number of infectious virions per unit volume in the concentrated supernatant.

The epithelial ductal structure in the murine mammary gland does not develop until 3-6 weeks after birth. Therefore it is possible to produce epithelium-free mammary glands by removing the epithelial rudiment at 2-3 weeks of age [16]. It has been demonstrated that mammary epithelial cells transplanted into such a "cleared" gland will form a ductal structure, and that the mammary

cells may be modified *in vitro* prior to implantation [16, 17]. Our results indicate that it is also possible to modify the epithelial rudiment itself by infection with a retrovirus.

Thirty mice (60 mammary glands) will be infected with either 2500 cfu (25 µl at 10^5 cfu/ml) of CA1 (β-gal only), CA1CR (expressing CR-1), or CA1CRZ (expressing a CR-1-specific ribozyme) in serum-free Dulbecco's modified Eagle's medium (DME) with 80 µg/ml polybrene; or treated with 25 µl DME plus 80 µg/ml polybrene alone. The infection will be performed by anaesthetizing the mouse, reflecting the skin to expose the #4 (inguinal) mammary gland, and injecting a 25 µl virus suspension into the gland. The wound will be closed with surgical clips, and aseptic technique will be maintained throughout as recommended by the WSU IACUC Guidelines for Survival Rodent Surgery. The use of both #4 glands will allow us to directly compare two treatments in the same mouse, any quantitative data may then be compared using a paired T-test; otherwise quantitative comparisons will be made among all groups using ANOVA and Fisher's PLSD. The initial chosen pairings will be CA1 with CA1CR and CA1CRZ with DME. If other specific pairings are warranted by the findings, the experiment can be repeated.

Three mice from each pairing (thus 3 glands from each treatment) will be sacrificed after 1 week, and every week thereafter for a total of 5 weeks. This schedule will allow us to follow the development of the mammary epithelium and evaluate the effect of the treatments throughout the process of gland development. In the future, it may be informative to examine the morphology of transduced glands in pregnant or aged mice, due to the higher observed levels of CR-1 expression in those glands. Changes in gland morphology may be qualitatively assessed by observation of the whole mounts. If desired, glands can be dissolved out of Permount with xylene and then embedded in paraffin for sectioning. Changes in CR-1 expression can be assessed in these sections by immunohistochemistry using a CR-1-specific antibody we have prepared against a synthetic peptide (first described in [10]).

As reported in the first year annual report, there were technical difficulties with the retroviral system chosen for the gene transduction experiments proposed throughout this grant application. Transduction of mammary epithelium was possible, but expression of the transgene was restricted to the duct end buds. It was not possible to make a completely transgenic epithelial tree. Thus, though SOW Tasks 1-3 were attempted with the retroviral vectors, no conclusive results were obtained.

The issue at hand is how to alter CR-1 expression or levels in the developing mammary gland. Since the retroviral strategy does not appear viable, we are left with choosing another vector (e.g. adeno-associated virus) or attempting to add CR-1 protein in some sort of slow-release system (e.g. Elvax pellet). There is one report [18] that implantation of a peptide consisting of only the EGF domain of CR-1 in Elvax pellets affected the growth and morphology of the developing epithelial tree. However, full-length native CR-1 protein has still not been isolated, and that may give different results than those observed with only a portion of the protein. Therefore, we chose to attempt to purify native CR-1 and add it back to the developing mammary gland. We had some success using immunoaffinity columns (second annual report), though purifying enough protein to use in vivo presented a problem. In the end, we could not purify enough protein to use for biological analysis.

One key open question regarding CR-1 is what proteins it interacts with on target cells. No receptor has been identified to date, though is has been established that CR-1 does not bind to the ErbB receptors [19, 20]. We were able to purify enough CR-1 to perform a far-western blot on mouse fibroblasts, mouse mammary epithelial cells, and human breast cancer cells. (Second annual report). No further progress was made on this avenue of research.

T.O. 1b. For precise quantification of CR-1 protein levels, the experiment will be repeated, and glands will be harvested for protein analysis by western blot. CR-1 will be detected using the same antibody as for immunohistochemistry.

This was not be performed, as we were unable to isolate enough CR-1 to use in slow-release pellets (SOW Task 4).

T.O. 2 We have designed and tested a hammerhead ribozyme [21, 22] that recognizes nucleotides 12-28 of the murine CR-1 mRNA and cuts after the GUC triplet at nucleotides 18-20. A search of the GenBank database (FASTA, GCG Wisconsin Package) revealed no significant nucleotide sequence identity to any other published sequence, including related EGF family members.

As described in the first year annual report (and published [23]), this ribozyme did not act through a catalytic mechanism. It is unclear why the ribozyme is not catalytic. Perhaps it is due to poor folding due to the long regions 5' and 3' of the ribozyme domain in the ribozyme transcript, or it may be due to the site chosen for ribozyme binding. During the course of investigation of the mechanism of ribozyme activity, we were able to obtain a vector for the expression of ribozymes which contains self-cleaving ribozymes which flank the ribozyme of interest 5' and 3' (kindly provided by J. Norris, University of South Carolina Medical School). We made two additional ribozymes which use this self-splicing system. The first targets the 5' untranslated region at residue 185, and the second targets the coding region at residue 519. We have tested the self-splicing ability of these two constructs and found it to be intact (second annual report).

The CL-S1 cell line was derived from a preneoplastic BALB/c mammary nodule, and the -SA, and +SA target cell lines were isolated from a spontaneous adenocarcinoma derived from the same nodule ([24]). CL-S1 cells are anchorage-dependent and do not form tumors in syngeneic hosts; -SA cells do not form colonies efficiently in soft-agar, but will form tumors in syngeneic hosts after injection *in vivo*; +SA cells display efficient colony formation *in vitro*, and are neoplastic and metastatic (pulmonary metastases after intravenous injection) when inoculated into syngeneic hosts *in vivo* [24]. CR-1 expression is strong in +SA cells, slightly lower in -SA cells, and low in CL-S1 cells (unpublished results). A CR-1 over-expression retroviral vector has also been constructed (CA1CR) and used to infect CL-S1.

T.O. 2a. Changes in the growth phenotype of the transfected clones which show the greatest difference in CR-1 levels from the parental line will be evaluated *in vitro* by examining growth rate and the relative efficiency of colony formation in soft agar [24] in triplicate cultures of the various

transfected lines. Differences in amount of cellular DNA and colony numbers in soft agar will be analyzed using ANOVA and Fisher's PLSD.

As stated in the first year's report, overexpression of CR-1 in CL-S1 cells did not cause any significant change in growth or ability to form colonies in vitro. Transfection of -SA cells with the original ribozyme was effective at reducing CR-1 expression, but neither overexpression nor underexpression affected growth on plastic or in soft agar. Transfection of +SA cells did affect growth in soft agar as shown in the second annual report. These experiments concluded Tasks 5-7.

T.O. 2b. The clones analyzed in T.O. 2a will be implanted into syngeneic mice for *in vivo* analysis. Five x 10^5 cells will be injected into epithelium-free (cleared) mammary fat pads of 6-8 week old female BALB/c mice. Six to eight week old animals will be used because (1) the fat pads are larger in these animals, so the surgical implantations are mechanically easier to perform; and (2) at three weeks of age, the mice are still in puberty, and we wish to minimize possible effects of the hormonal state of the animal on the results. The mice will be palpated twice per week for tumor development and tumor size will be measured with a caliper. In untreated mice, +SA-derived tumors are typically palpable after 14-21 days [24]. Therefore, mice will be sacrificed at the end of 4 weeks, unless they are moribund prior to that time, in which case they will be sacrificed immediately and the date of sacrifice noted. Mammary tumors will be further evaluated after fixation, clearing and staining of whole mammary glands. Both whole mounts and sections from them will be analyzed with respect to outgrowth pattern of the transplanted cells (normal vs. hyperplastic). Differences in tumor size (and number if separate tumors are generated) will be compared among all cell types by ANOVA and Fisher's PLSD.

The same cell populations (1 x 10^5 cells) will be injected into the tail vein of 6-8 week old BALB/c mice to evaluate the potential for the formation of pulmonary metastases [24]. Three mice will be used for each of the cell populations, and they will be sacrificed at 4 weeks after inoculation. Differences in pulmonary tumor number and size will be analyzed using ANOVA and Fisher's PLSD.

In the first annual report we discussed the transfer of -SA/pRZECR-1 clones into mice. It is still not clear if the tumors observed were due to reversion of these cells or recruitment of other cells into the tumors (all of which were CR-1-positive). No further experiments were conducted on this technical objective.

T.O. 3a. Five x 10^5 +SA cells will be implanted into the cleared #4 fat pads of sixteen 6-8 week old mice. The position of the +SA cells in the gland will be marked on the skin surface to facilitate later injection of viral suspensions. The glands will be infected with 2 x 10^6 cfu (in 2 injections of 25 µl at 4 x 10^4 cfu/µl) of the β-gal-expressing CA1 retroviral vector in the presence of 80 µg/ml polybrene [27]. Injections will begin from 0-14 days after the implantation of the tumor cells and will be repeated every other day until day 18 (see Table 3 for a summary). All mice will be sacrificed on day 28 after implantation of tumor cells and the glands will be removed for analysis. Glands will be fixed and stained for β-gal. Tumor size, percentage of infected (β-gal positive) tumor cells, percentage of infected surrounding cells, and distance of viral spread from the

injection point will be recorded. The optimal time after implantation of the tumor cells and the maximum time after implantation (and therefore maximum tumor size) allowing efficient infection with the vector will be determined. The planned viral inoculum will result in a vector: target ratio of 4:1 at the time of tumor cell implantation, if all of the transplanted tumor cells survive. Should these vector:target ratios prove insufficient for efficient infection of the tumor, we can either reduce the initial tumor cell dose or attempt to further increase the viral titer.

SOW Tasks 9 and 10; these experiments have not yet begun and due to problems with the retroviral constructs will not be attempted as written.

T.O. 4a. The retroviral constructs CA1 and CA1CRZ used in T.O. 1 will be used to treat +SA tumors *in vivo*. +SA tumors will be generated in the cleared #4 mammary fat pads of fifteen 6-8 week old mice as described in T.O. 3. Ten of the mice will be infected with CA1 and CA1CRZ at the optimal conditions determined under T.O. 3 (CA1 on one side CA1CRZ on the other). The five remaining mice will receive only DME containing 80 μg/ml polybrene (in both sides) as a control (see Table 4 for a summary). This design will allow a paired comparison (controlling for host variation) between CA1 and CA1CRZ, and will allow a general comparison (ANOVA + Fisher's PLSD) among all three groups. Glands will be scored for tumor size, percentage of infected tumor cells, and percentage of infected surrounding cells (determined by β-gal staining). A group size of ten (glands) for each treatment is used here rather than the six used in T.O. 2, because reliance on infection of the tumor cells rather than using a stably transfected tumor cell line may increase experimental variability.

SOW Task 11; these experiments were not performed, as even the new vectors did not appear to alter CR-1 expression.

New vectors: We cloned the murine and human CR-1 genes, the human CR-3 gene, and position 185 and 519 (murine) self-splicing ribozymes into the pAdTrackCMV shuttle vector for use with the AdEasy system developed in Vogelstein's laboratory [25]. The ribozymes did not appear to work in this context, perhaps due to different compartmentalization of the RNA than is seen with transfection, perhaps due to some effect of infection with the adenovirus. This essentially ended any further work on the murine CR-1 gene.

During the construction of the adenoviral vectors and the attempts to purify CR-1 from MCF-7 cells, we serindipitously cloned the human CR-3 gene from MCF-7 breast cancer cells. We inserted this gene into our adenoviral expression system, and found a profound difference in the effect of CR-1 versus CR-3 expression in epithelial cells. It is important to note that these differences are in the context of the expression of GFP by our vector (which appears to substantially stress the epithelial cells), and we are currently making GFP-free vectors to repeat these experiments. We have described these results in a manuscript entitled "

However, some very interesting results were obtained with the human CR-1 and CR-3 viruses. We have submitted a manuscript and proposed future experiments (USAMRMC IDEA proposal June 2000) based on these findings.

Current findings on the potential functional regions of Cripto

The human Cripto-1 (CR-1) gene was identified in 1989, and is considered a member of the epidermal growth factor family of ligands due to sequence homology. The protein was later associated with breast (80%), pancreatic (45%), gastric (50%), and colon (80%) tumors, among others. In 1991, a very similar gene named CR-3 was identified that has several modifications including six amino-acid changes. CR-3 appears to be the result of a retroposition event, due to its lack of introns. We have cloned CR-3 from MCF-7 cells and 293 cells using PCR. The cDNA clone of CR-1 and DNA clone of CR-3, both including full 5'- and 3' UTR regions, were inserted into adenoviral expression vectors with CMV promoters. This vector system also includes a GFP gene running from a second CMV promoter. MCF-7 cells infected with the Ad/CR-1 construct show increased message but not increased protein production, while infection with Ad/CR-3 produces message and a substantial alteration of the Cripto protein profile as detected by Western analysis. There are also morphological and growth changes in MCF-7 and Vero cells infected with control and treatment vectors. For both cell types, infection with a GFP-only vector initially causes a suppression of growth, and later results in cell death that is probably GFP-induced apoptosis. The CR-1 construct is more inhibitory to initial cell growth than is the empty vector but delays the onset of cell death. The CR-3 construct is more inhibitory to growth than is the control vector in Vero but not MCF-7 cells, but in both cases shortens the time taken for cell death. These data suggest 1) that CR-1 production and activity are tightly regulated in cells, and 2) that the differences between CR-1 and CR-3 can be exploited to provide clues about the functional domains of Cripto.

Figure 1A compares the published CR-3 sequence (Ac. # M96956, Dono et al., 1991) to our CR-3 sequence (obtained from both MCF-7 and 293 cells, Ac. #s AF251549 and AF251550) and the sequence of the human PAC clone DJ302C05 of the Xq23 region (Ac. # AC000113). There are some minor differences between our sequence and the published sequence for CR-3; however there is 100% identity between our sequence and the PAC sequence. Figure 1B compares the predicted amino acid sequence of CR-3 against the predicted amino acid sequence of CR-1. There are only six amino acid changes between the proteins.

A

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CR-3 (pub.)     1  GGAGAATCCCCGGAAAGGCTGAGTCTCCAGCTCAAGGTCAAAACGTCCAAGGCCGAAAGCCCTCCAGTTTCCCCT   75
CR-3 (MCF-7)
Xq23 PAC clone

                76  GGACACCTTGCTCCTGCTTCTGCTACGACCTTCT.GGGAACGCGAATTTCTCATTTTCTTCTTAAATTGCCATTT   150
                                                      G
                                                      G
               151  TCGCTTTAGGAGATGAATGTTTTCCTTTGGCTGTTTTGGCAATGACTCTGAATTAAAGCGATGCTAACGCCTCTT   225
               226  TTCCCCCTAATTGTTAAAAGCTATGGACTGCAGGAAGATGGTCCGCTTCTCTTACAGTGTGATTTGGATCATGGC   300
               301  CATTTCTAAAGCCTTTGAACTGGGATTAGTTGCCGGGCTGGGCCATCAGGAATTTGCTCGTCCATCTCGGGGAGA   375
               376  CCTGGCCTTCAGAGATGACAGCATTTGGCCCCAGGAGGAGCCTGCAATTCGGCCTCGGTCTTCCCAGCGTGTGCT   450
               451  GCCCATGGGAATACAGCACAGTAAGGAGCTAAACAGAACCTGCTGCCTGAATGGGGGAACCTGCATGCTGGAGTC   525
               526  CTTTTGTGCCTGCCCTCCCTCCTTCTACGGACGGAACTGTGAGCACGATGTGCGCAAAGAGAACTGTGGGTCTGT   600
               601  GCCCCATGACACCTGGCTGCCCAAGAAGTGTTCCCTGTGTAAATGCTGGCACGGTCAGCTCCGCTGCTTTCCTCA   675
               676  GGCATTTCTACCCGGCTGTGATGGCCTTGTGATGGATGAGCACCTCGTGGCTTCCAGGACTCCAGAACTACCACC   750
               751  GTCTGCACGTACTACCACTTTTATGCTAGCTGGCATCTGCCTTTCTATACAAAGCTACTATTAATCGACATTGAC   825
               826  CTATTTCCAGAAATACAATTTTAGATATTATGCAAATTTCATGACCCGTAAAGGCTGCTGCTACAATGTCCTAAC   900
               901  TGAAAGATGATCATTTG.TAGTTGCCTTAAAATAATGAATACAATTTCCAAAACGGTCTCTAACATTTCCTTACA   975
                                T                                  .
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                        T                                .
 976  GAACTAACTACTTCTTACCTCTTTGCCCTGCCCTCTCCCAAAAAACTACTTCTTTTTTCAAAAGAAAGTCAGCCA   1050
1051  TATCTCCATTGTGCCCAAGTCCAGTGTTTCTTTTTTTTTTTTGAGACGGAGTCTCACTCTGTCACCCAGGCTGGA   1125
1126  CTGCAATGACGCGATCTCGGTTCACTGCAACCTCCGCATCCGGGGTTCAAGCCATTCTCCTGCCTCAGCCTCCCA   1200
1201  AGTAGCTGGGATTACAGGCATGTGTCACCATGCC.GGCTAA.TTTTTTTGTA.TTTTAGTAGAGACGGGGGTTTC   1275
                                        C      T          T
                                        C      T          T
1276  ACCATATTGGCCAG.CTGGTCTCGAACT.CTGACCTTGTGATCCA.TCGC..TCGCCTCTCG.AGTGCTGAGATT   1350
                    C             C               C    CTCG        A
                    C             C               C    CTCG        A
1351  ACACACGTGAGCAACTGTGCAAGGCCTGGTGTTTCTTGATACATGTAATTCTACCAAGGTCTTCTTAATATGTTC   1425
                                           G
                                           G
1426  TTTTAAATGATTGAATTATACACTCAGATTATTGGAGACTAAGTCTAATGTGGACCTTAGAATACAGTTTTGAGT   1500
                      C TCT          AA       T
                      C TCT          AA       T
1501  AGAGTTGATCAAAATCAATTAAAATAGTCTC.TTTAAAAGGAAAGAAAACATCTTTAAGGGGAGGAACCAGAGTG   1575
                                    T
                                    T
1576  CTGAAGGAATGGAAGTCCATCTGCGTGTGTGCAGGGAGACTGGGTAGGAAAGAGGAAGCAAATAGAAGAGAGAGG   1650
1651  TTGAAAAACAAAATGGGTTACTTGATTGGTGATTAGGTGGTGGTAGAGAAGCAAGTAAAAAGGCTAAATGGAAGG   1725
1726  GCAAGTTTCCATCATCTATAGAAAGCTATGTAAGACAAGGACTCCCCTTTTTTTCCCAAAGGCATTGTAAAAAGA   1800
1801  ATGAAGTCTCCTTAGAAAAAAAATTATACCTCAATGTCCCCAACAAGATTGCTTAATAAATTGTGTTTCCTCCAA   1875
1876  GCTATTCAATTCTTTTAACTGTTGTAGAAGAGAAAATGTTCACAATATATTTAGTTGTAAACCAAGTGATCAAAC   1950
1951  TACATATTGTAAAGCCCATTTTTAAAATACATTGTATATATGTGTATGCACAGTAAAAATGGAAACTATATTGAC   2025
                                           C
                                           C
2026  CT  2027
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B

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CR-1          1  MDCRKMARFSYSVIWIMAISKVFELGLVAGLGHQEFARPSRGYLAFRDDSIWPQEEPAIRPRSSQRVPPMGIQHS   75
CR-3 (MCF7)         V              A                    D                          L
             76  KELNRTCCLNGGTCMLGSFCACPPSFYGRNCEHDVRKENCGSVPHDTWLPKKCSLCKCWHGQLRCFPQAFLPGCD   150
                       E
            151  GLVMDEHLVASRTPELPPSARTTTFMLVGICLSIQSYY*  189
                                    A
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Fig. 1. A, comparison of the published sequence for CR-3 (Dono et al., 1991; Ac. # M96956) with the sequence obtained via PCR from both MCF-7 (Ac. # AF251549) and 293 (Ac. # AF251550), and clone DJ302C05 of the Xq23 region of the human genome (Ac. # AC000113). B, comparison of the putative amino acid sequences for CR-1 (Ac. # XP_002787) and CR-3, showing the six amino acid differences between the proteins.

We used Western analysis to assay for successful overexpression of CR-1 and CR-3. Figure 2A is a Western blot showing the protein profiles for uninfected MCF-7, as well as MCF-7 infected with control and overexpression vectors. There was minimal alteration in protein profile associated with infection (lane 2). As compared to the infected control, the only effect seen due to the Ad/CR-1 vector was a reduction in the intensity of the 38 kDa band (lane 3). The Ad/CR-3 construct, on the other hand, produced a shift in one protein band from 58 kDa to 51 kDa (lane 4). Densitometry measures of band intensity are shown in Fig. 2B.

A.

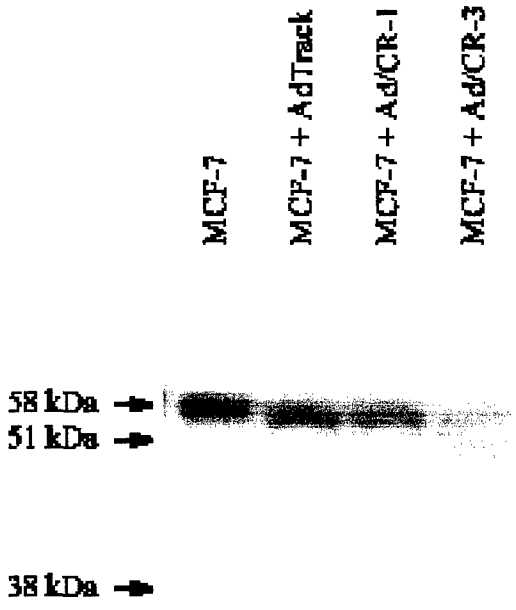


Fig. 2. A, Western blot of lysates of MCF-7 cells which were infected with various adenoviral vectors that included GFP as a tracking molecule.

B.

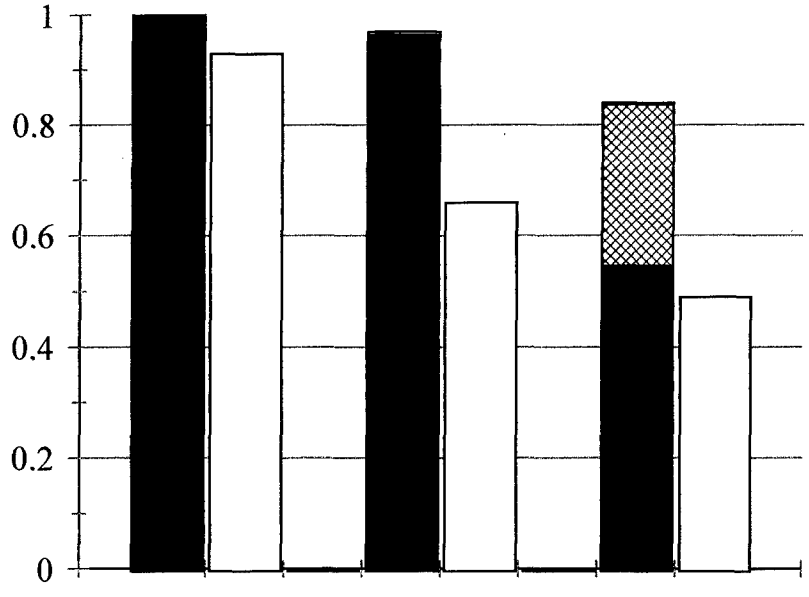


Fig. 2 B, densitometry measurements of the blot in A. Height of the bars reflects the ratio of the given band to the comparable band from the uninfected lysate; both 58 and 51 kDa bands were compared to the 58 kDa band from uninfected MCF-7s. Black bars indicate the 58 kDa bands, white bars represent the 38 kDa bands, and the stippled bar represents the 51 kDa band and is stacked on the black bar to allow easy comparison of the total amount of protein present in each lane.

Analysis of infected cells via RT-PCR did not detect message for Cripto proteins in either uninfected or AdTrack-infected lysates. However, message was detected in cell lysates from both the Ad/CR-1 and Ad/CR-3 infected cells, with the CR-3 construct producing a higher signal (Fig. 3).

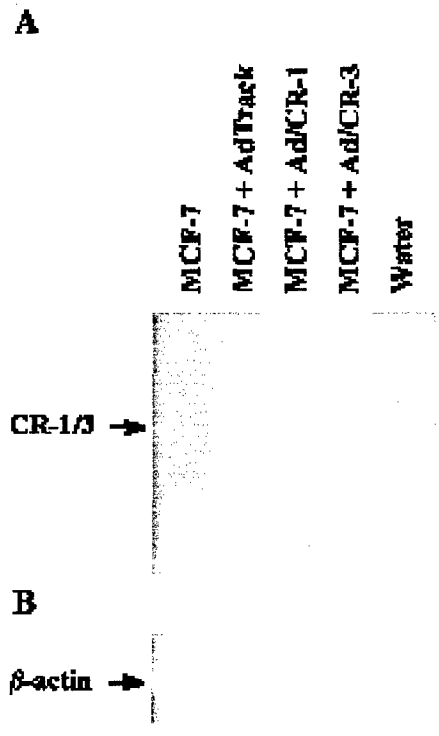


Fig. 3. A, ethidium bromide-stained agarose gel of RT-PCR reactions using RNA isolated from MCF-7 cells infected with various adenoviral vectors, and Cripto-specific primers. The predicted message size of 2 kb is the same for both CR-1 and CR-3. B, RT-PCR of identical RNA samples as above, but using primers specific for human β-actin.

MCF-7 and Vero cells infected with GFP-containing viruses fluoresced green when exposed to blue (488 nm) light, as would be expected of cells producing GFP (Figs. 4 and 5, panels D, F, and H). Uninfected control cells of both types did not fluoresce (Figs. 4 and 5, B). Vector-only treatments showed a number of cells with an atypical rounded phenotype which were extremely fluorescent (Figs. 4 and 5, C and D), and though there were still many adherent Vero cells, these cells looked "fatter" than uninfected cells (Fig. 5, A). In MCF-7 cells, the Ad/CR-1 vector resulted in cells with various intensities of green (Fig. 4, F). The cells which fluoresced the most brightly showed the atypical phenotype seen in the vector-only control. In comparison with the white-light image (Fig. 4, E), it is clear that many of the MCF-7 cells escaped infection by this construct or shut down expression from the CMV promoter, as these cells had normal morphology. In Vero cells, the Ad/CR-1 produced intensely fluorescent cells, most of which were adherent (Fig. 5, E and F). When MCF-7 cells were treated with the Ad/CR-3 construct, nearly all cells were infected, and they were rounded and detached from the plate surface (Fig. 4, G and H). Vero cells showed a more extreme morphology when treated with the Ad/CR-3 construct. At Day 6, fewer than 5% of cells were still adherent and the rounded cells had begun to disintegrate around the edges (Fig. 5, G).

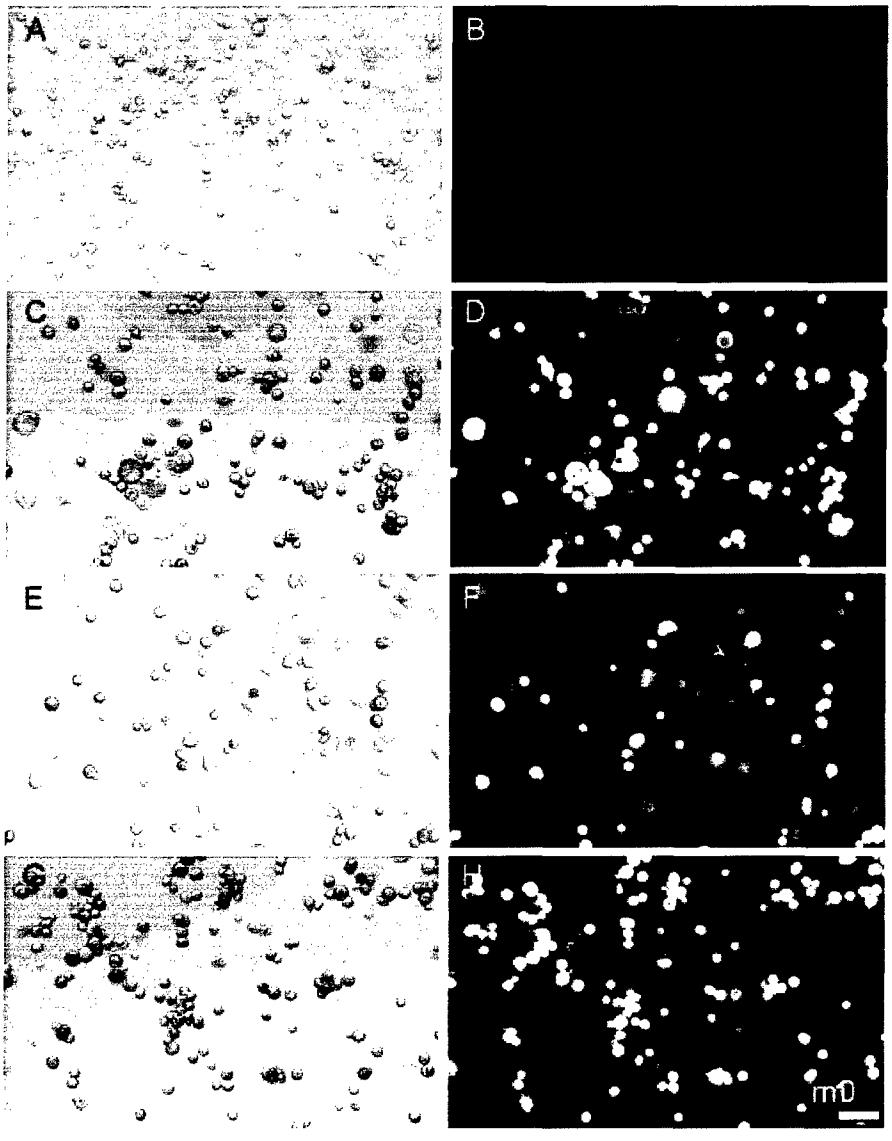


Fig. 4. White light (panels A, C, E, and G) and fluorescent (panels B, D, F, and H) confocal micrographs of uninfected (A & B), and AdTrack (GFP only) (C & D), Ad/CR-1 (GFP+CR-1) (E & F), and Ad/CR-3 (GFP+CR-3) (G & H) treated MCF-7 cells taken 6 days after infection. Images for each treatment show identical fields of view. The scale bar is equal to 0.1 mm.

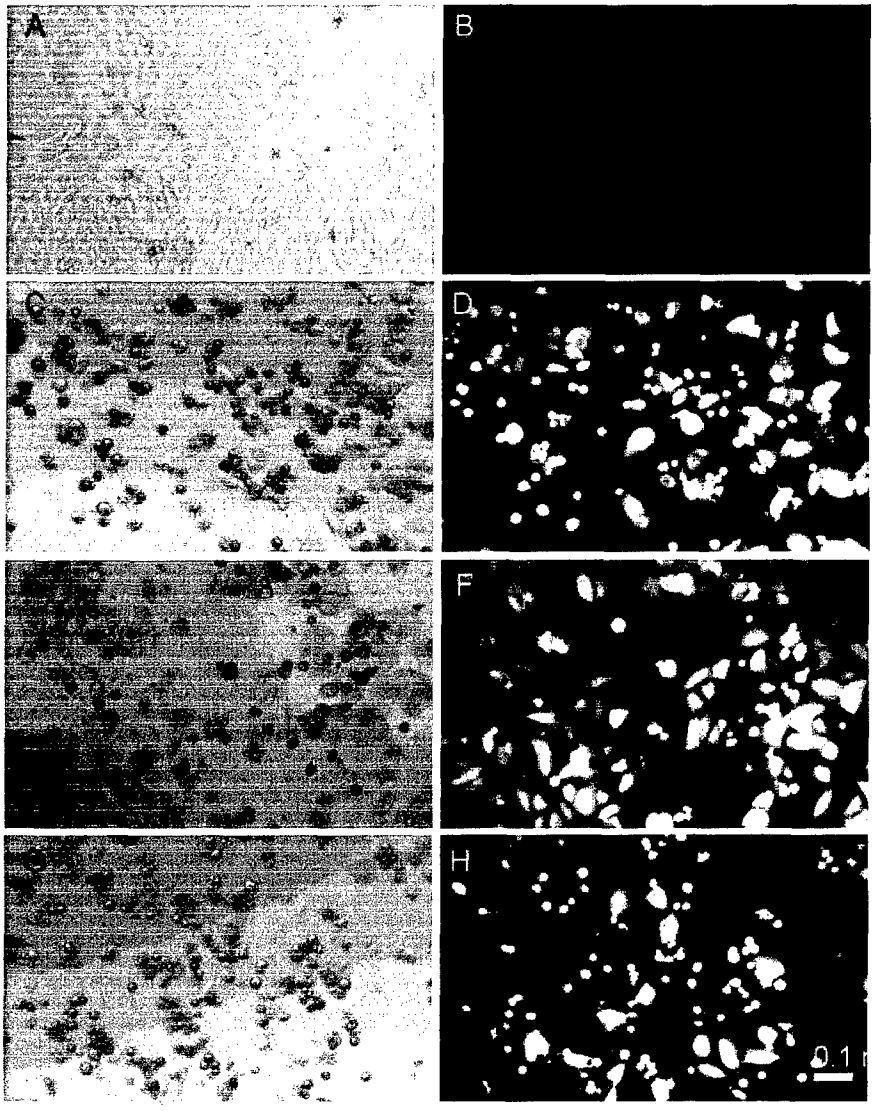
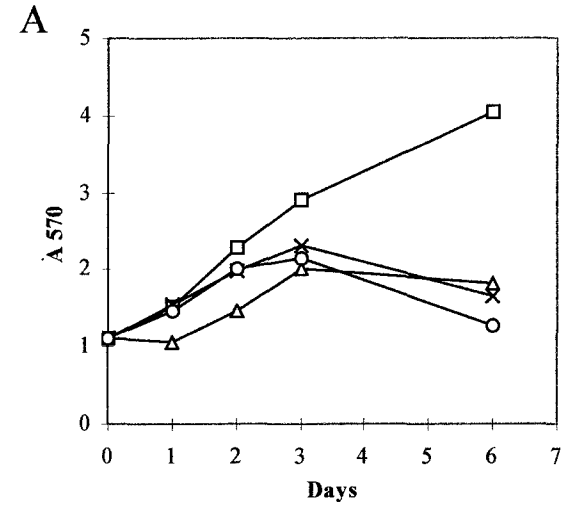
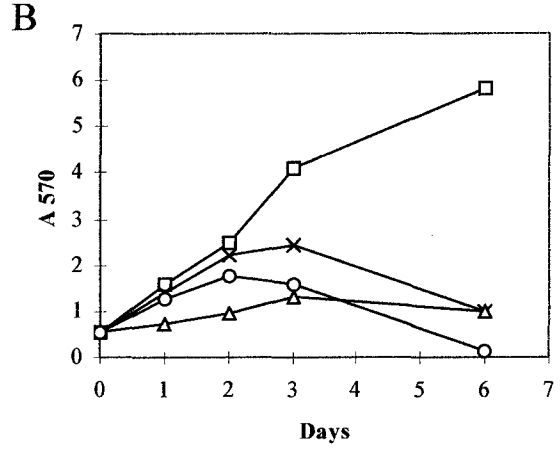


Fig. 5. White light (panels A, C, E, and G) and fluorescent (panels B, D, F, and H) confocal micrographs of uninfected (A & B), and AdTrack (GFP only) (C & D), Ad/CR-1 (GFP+CR-1) (E & F), and Ad/CR-3 (GFP+CR-3) (G & H) treated Vero cells taken 6 days after infection. Images for each treatment show identical fields of view. The scale bar is equal to 0.1 mm.

Growth curves performed on both MCF-7 and Vero cells produced similar results (Fig. 6A and B). As seen in the previous figures, there was a strong effect of infection. While some authors using adenoviral vectors with GFP for tracking have attributed a similar effect to the use the virus [26], GFP itself has been shown to cause apoptosis in cells within 36 hours of transfection with GFP-containing plasmids [27]. In spite of this complication, CR-1 and CR-3 constructs both appeared to reduce the amount of initial growth relative to the (GFP+) vector-only control. In addition, CR-1 delayed the point at which cells began to die, while CR-3 resulted in more-rapid death than the (GFP+) vector-only control.

We also conducted a growth curve in HC-11 cells (mouse mammary epithelial line) and found that they were more resistant to the apparent toxic effects of GFP. In this case, we found that infection with the CR-1-expressing vector caused an increase in growth relative to the GFP-expressing control. However, similar to the Vero and MCF-7 cells, the CR-3-expressing vector grew less well than GFP-expressing controls. All of these data are consistent with a growth/protective effect of CR-1 that is not conferred by CR-3.





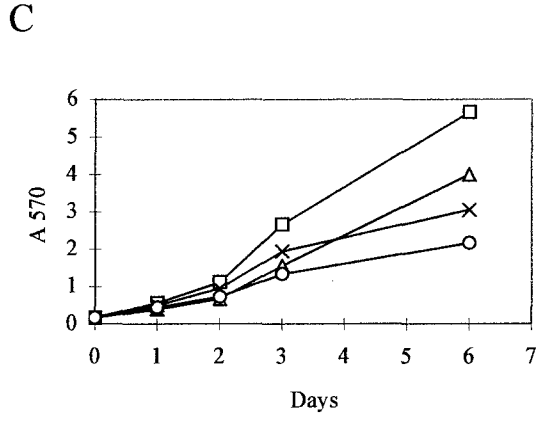
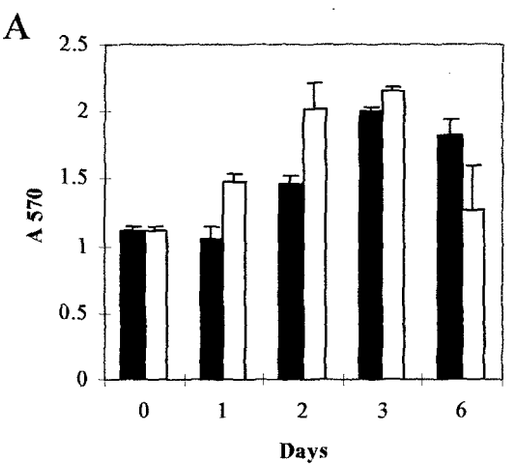


Fig. 6. Growth curves of MCF-7 (A), Vero (B), and HC-11 (C) cells which have been infected with various adenoviral constructs as measured by MTT assay. The y-axis shows A570 nm averaged for data collected in triplicate. Uninfected control (-□-); AdTrack control (-x-); Ad/CR-1 (-Δ-); Ad/CR-3 (-○-).

The differences in growth pattern between Ad/CR-1 and Ad/CR-3 treated, GFP-sensitive cells are highlighted in Fig. 7A and B. The data show that Ad/CR-1 treated cells grew more slowly than the Ad/CR-3 treated cells, but the Ad/CR-3 treated cells decline precipitously beginning at day 3 of the curve.



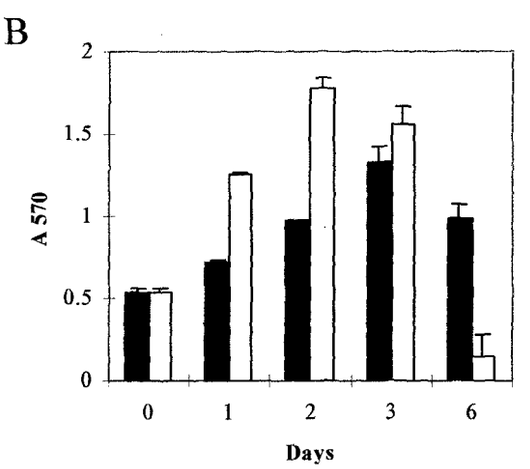


Fig. 7. Bar graphs highlighting differences in growth characteristics of MCF-7 (A) and Vero (B) cells caused by infection with Ad/CR-1 (black bars) and Ad/CR-3 (white bars).

The difficulty of working with CR-1 is made evident by the fact that it was identified more than 10 years ago, associated with a transformed phenotype in the initial description [2], and a shorter-than-full-length recombinant protein has only recently been isolated [28]. Full-length native protein has yet to be isolated. In the studies funded by this final year of our grant, we have shown that substantial overexpression of CR-1 message does not result in a corresponding increase in protein as detected via Western blot. These results indicate that the production of CR-1 protein is tightly regulated at some point after transcription. It may be that one problem with isolation of native protein is tight post-transcriptional regulation of the protein. Tight regulation, at multiple stages of expression, is

characteristic of oncogenic proteins [29]. This matches closely with the initial observations that overexpression of CR-1 in cells which do not normally produce the protein causes a transformed phenotype [2, 12].

It is interesting to note that in spite of the minor alterations in protein profile generated by the "overexpression" of CR-1, there were substantial changes in cell morphology as compared to vector-only controls. Our data indicate that although CR-1 slowed the growth of Vero and MCF-7 cells, it also may have reduced the rate of apoptosis. In HC-11 cells, CR-1-tranduced cells actually grew better than GFP-expressing controls. This is in direct contrast to the effect of CR-3, which enhanced the rate of cell death in all cell types. We are currently exploring this phenomenon further. These studies will be facilitated by the construction of GFP-free vectors; however the interaction of CR-1 and CR-3 with an apoptosis-inducing factor provides interesting information about the possible functions of Cripto proteins.

CR-3 is a naturally-occurring variant of Cripto protein. It is unclear whether or not CR-3 is normally expressed in any cells. One complicating factor in the CR-1/CR-3 story is that methods involving nucleic acid hybridization (e.g. in situ hybridization, Northern blotting) or antibody detection (e.g. immunostaining or Western blotting) are unlikely to distinguish between the nucleic acids or proteins. The studies relating CR-1 to cancers have all relied on these techniques. While it is clear that some Cripto protein is certainly associated with human cancers, it has yet to be demonstrated which one. This leaves open the possibility that CR-3 may, in fact, be an important player in tumorigenesis in its own right, though this has yet to be demonstrated.

One of the differences between CR-1 and CR-3 is the lack of a tyrosine at position 43 in the latter, which may result in differential regulation of the two proteins. This is one of just six amino acids that vary between the CR-1 and CR-3 proteins. One or all of these differences may be responsible for the differing cellular phenotypes which result from their overexpression. Using site-directed mutagenesis, we can determine which regions of the proteins are critical for producing these phenotypes, and come to a greater understanding of the role of Cripto proteins in human cancer. We have currently submitted an IDEA proposal to the USAMRMC to conduct these experiments and have received some limited start-up funding to begin this work from WSU.

Key Accomplishments

1. It is possible to eliminate CR-1 protein expression through the use of a ribozyme-like molecule, even without catalytic activity. This decrease is substantially better than that previously observed with antisense strategies [13]. This may indicate that RNA molecules with strong secondary structure can be targeted to the extreme 5' end of a message to obtain excellent reduction or elimination of expression.

2. Overexpression of Cripto-1 in the preneoplastic line CL-S1 did not cause an increase in growth, colony formation in soft agar, or tumor formation *in vivo*.

3. Elimination of CR-1 expression in -SA cells did not affect growth rate or colony formation *in vitro*. The *in vivo* results are not yet clear.

4. It is possible to transduce nascent mammary epithelium with injection of either retrovirus or retrovirus-producing cells. However, expression appears to be restricted to actively dividing cells, not the entire epithelial tree.

5. It is possible to significantly alter the ability of +SA cells to form colonies in soft agar by transfecting them with CR-1 overexpression and underexpression vectors. The ribozyme vector appeared to work slightly better than the antisense vector. The retroviral vector inhibited colony formation with or without CR-1 expression.

6. It is possible to purify CR-1 using an antibody affinity column, though the amount of protein purified must be increased to be useful as a reagent.

7. Use of the purified CR-1 in a far-western blot showed that there are at least two CR-1 binding proteins present in human and mouse mammary epithelial cells.

8. We have developed at least one more CR-1-specific ribozyme. This one will cleave CR-1 transcripts in vitro.

9. Human CR-1 and CR-3 have differing effects on the growth and survival of epithelial cells in culture.

10. As CR-1 and CR-3 differ by only 6 amino acids, this provides a strong indication of potential functional domains in Cripto. No experiments to date have even addressed the potential functional regions of Cripto.

11. We have constructed site-directed mutants in the 6 amino acid differences between CR-1 and CR-3 to determine which residue or residues are important for gene function. Future funding will allow us to proceed with these experiments.

Reportable Outcomes

1. Kintner, R.L. and Hosick, H.L. 1998. Reduction of Cripto-1 expression by a hammerhead-shaped RNA molecule results from inhibition of translation rather than mRNA cleavage. *Biochem. Biophys. Res. Comm.* **245:**774-779.

2. Kintner, R.L., M.A. Kintner, and H.L. Hosick. 1999. Generation of Recombinant Adenoviruses to Modify Cripto-1 Expression. Presented at the Inland Northwest Cancer Conference.

3. Kintner, M.A., R.L. Kintner, and H.L. Hosick. 2000. Human Cripto-1 and Cripto-3 have different effects on the growth characteristics of MCF-7 and Vero cells. Presented at the Era of Hope Department of Defense Breast Cancer Research Program Meeting.

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